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Research Article

Triple arginines in the cytoplasmic tail of endomannosidase are not essential for type II membrane topology and Golgi localization

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Keywords. Endomannosidase, glucose-trimming, *N*-linked oligosaccharides, Golgi apparatus, Golgi retention.

Introduction

Newly synthesized proteins contain targeting signals consisting of short discrete amino acid sequence motifs that direct them to distinct compartments within the cell. Such sequences within proteins were identified for their targeting or retention in endosomes, lysosomes and endoplasmic reticulum (ER) (for review see [1–3]).

Various signals for retention and/or retrieval in the ER have been identified. Type I membrane proteins are retained in the ER by a di-lysine motif correctly positioned relative to the C terminus [4–7], whereas an N-terminal double arginine motif in the cytoplasmic tail of type II membrane proteins might serve as ER targeting signal [8]. On the other hand, soluble ER proteins containing the C-terminal KDEL/HDEL tetrapeptide [9, 10] are efficiently retrieved from the Golgi to the ER by the KDEL receptor [11]. Prior to ER retrieval *via* a retrograde transport route, these proteins receive a Golgi-associated post-translational modification [6, 12].

Various glycosidases and glycosyltransferases are localized in distinct partially overlapping subcompartments of the Golgi apparatus [13] possessing a type II

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membrane architecture [14]. Although they share a common domain structure, no specific targeting signals for Golgi localization could be identified. There is evidence that not only the transmembrane domain but also the cytoplasmic and/or luminal sequences play a role for Golgi retention [15–19]. Furthermore, cell type-related variability has been observed for the same Golgi enzyme in regard to the role of domains for retention [20]. Two models have been proposed to explain Golgi retention. In the kin recognition model [21], it was suggested that Golgi enzymes interact with each other forming large hetero-oligomeric structures that are too large to enter transport vesicles. In the lipid bilayer model, the hydrophobic transmembrane region is proposed to retain the enzyme in the Golgi apparatus [15].

Endomannosidase represents a unique endoglycosidase in the *cis* and *medial* Golgi apparatus [22, 23] and provides there an alternate glucosidase-independent glucose-trimming pathway. The mechanism by which the enzyme is retained in the Golgi apparatus is currently unknown. Cloning of endomannosidase [24–26] revealed a high interspecies amino acid sequence identity [26]. Endomannosidases share particular features, *i.e.*, three consecutive positively charged amino groups close to the N terminus followed by a single stretch of hydrophobic amino acids (residues 10–25) and conserved amino acid regions close to the C terminus [26]. It is not known whether the hydrophobic region is part of an uncleavable 25-amino acid signal sequence and functions as a membrane-spanning domain resulting in a type II membrane protein topology. Alternatively, a soluble enzyme could be generated by a cleavable signal sequence or if one of the two conserved methionine residues at position 23 and 24 [26] serves as an alternative translation start.

In this study, we report the molecular architecture of endomannosidase and demonstrate that both its cytoplasmic and transmembrane domains are required for its efficient Golgi localization. Furthermore, we demonstrate that the positively charged amino acids in the cytoplasmic tail are not essential for type II membrane topology of the enzyme.

Materials and methods

Materials. Rabbit anti-human Sec61β antibody was kindly provided by Dr. B. Dobberstein (University of Heidelberg, Heidelberg, Germany) and rabbit anti-Golgi mannosidase II antibody by K. Moremen (Complex Carbohydrate Research Center, Athens, GA). Rabbit anti-human α1-antitrypsin antibody was purchased from Dako (Zug, Switzerland). The mouse monoclonal anti-myc antibody was from Upstate (Milton Keynes, UK). Rabbit anti-green fluorescent protein (GFP), rhodamine-conjugated $F(ab')_2$ fragments of goat anti-rabbit IgG and Alexa 488-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG were from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Restriction enzymes, T4 DNA ligase, endoglycosidase H (Endo H), recombinant *N*-glycosidase *F* (PNGase F) and protease inhibitor tablets were purchased from Roche Diagnostics (Rotkreuz, Switzerland), Taq DNA polymerase, expression vectors pcDNA3.1, pcDNA6/myc-His, pEGFP vector, competent *E. coli* DH5a cells, Lipofectamine 2000, cell culture media and fetal bovine serum from Invitrogen (Basel, Switzerland) and QIAquick PCR-purification Kit from Qiagen (Basel, Switzerland). Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland).

Recombinant DNAs. For chimeric α1-antrypsin ($rEndo_{25}$/A1AT, Fig. 1A) the signal sequence of human α1-antitrypsin comprising the amino acids 1–24 was replaced with the DNA fragment encoding the 25 amino acids of the putative signal sequence of rat endomannosidase using PCR amplification. Full-length rat endomannosidase subcloned in the expression vector pcDNA6 [27] served as template in combination with the corresponding sense/antisense oligonucleotides (Table 1).

For construction of rat endomannosidase with truncations in the signal sequence (Fig. 1B and C), or the replacement of the transmembrane domain by polyleucines (Fig. 1A) as well as for constructs containing mutations in the cytoplasmic tail (Fig. 1D) full-length rat endomannosidase cDNA in pcDNA6 vector [27] was used as template in the PCR amplification in combination with the corresponding sense/antisense oligonucleotides (Table 1). The corresponding PCR products were either subcloned into the *Hin*d III/*Xho* I site of the pcDNA6 vector in frame with the myc-tag or into the *Hin*d III/*Bam* HI site of the pEGFP vector for rat $endomannosidase_{25}$. The correctness of each construct was verified by DNA-sequencing.

Cell lines and transfections. Clone 9 hepatocytes, CHO-K1, HepG2 and HeLa cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in Ham's F12 medium with 10% FBS. Transfections of various cell lines with different constructs were performed using Lipofectamine

Table 1. Oligonucleotides used for the generation of chimeric α1-antitrypsin (A1AT), and GFP constructs and various rat endomannosidase mutants[a].

Primer	5′-3′ sequence
Chimeric-A1AT (s)	TAG**AAGCTT**GTCATCATGGCAAAATTCCGAAGAAGGACCTGCAT
Chimeric-A1AT (as)	**GGATCC**TCTAAGCCCATCATCAGAGAGAAA
$rEndo_{23}$-GFP (as)	**GGATCC**CATCAGAGAGAAAAT
$rEndo_{21}$-GFP (as)	**GGATCC**AGAGAAAATAAATAC
$rEndo_{19}$-GFP(as)	**GGATCC**AATAAATACAATAAA
$rEndo_{17}$-GFP (as)	**GGATCC**TACAATAAAAAGTGA
$rEndo_{15}$-GFP (as)	**GGATCC**AAAAAGTGACAAAAT
$rEndo_{13}$-GFP (as)	**GGATCC**AAAAGTGACAAAAT
$rEndo_{11}$-GFP (as)	**GGATCC**AATGATGCAGGTCCT
$rEndo_{9}$-GFP (s)	AGCTTGTCATCATGGCAAAATTCCGAAGAAGGACCTGCG
$rEndo_{9}$-GFP (as)	GATCCGCAGGTCCTTCTTCGGAATTTTGCCATGATGACA
Δsig-rEndo (s)	TAG**AAGCTT**GTCATCATGGCAAAATTCCGAAGAAGGACCTGCAT
ΔCT-rEndo (s)	**AAGCTT**GTCATCATGATCATTTTGTCACTTTTTATTGTATTTATTTTCT
ΔTMD-rEndo (s)	**AAGCTT**GTCATCATGGCAAAATTCCGAAGAAGGACCTGCTTAAAGATGCTGTGG
rEndo ΔTMD/L_{16}	TAG**AAGCTT**GTCATCATGGCAAAATTCCGAAGAAGGACCTG*CCTCCTCCTGCTGTTGCTTTTGC TCCTGCCCTGCTTCTTCTGCTGCTCTTA*AAGATGCTGTGGCC
rEndo ΔTMD/L_{23}	TAG**AAGCTT**GTCATCATGGCAAAATTCCGAAGAAGGACCTG*CCTCCTCCTGCTGTTGCTTTTGC TCCTGCTCCTGCTTCTTCTGCTGCTCCTCTTGCTACTGCTTCTCCTCTTA*AAGATGCTGTGG
rEndo R_{5-7}K (s)	**AAGCTT**GTCATCATGGCAAAATTC*AAGAAAAAG*ACCTGCATCATTTTGTCACT
MRRR-rEndo (s)	TAG**AAGCTT**GTCATCATG*CGAAGAAGG*ACCTGC
MLLL-rEndo (s)	TAG**AAGCTT**GTCATCATG*CTACTGCTG*ACCTGC
rEndo (as)	T**CTCGAG**CGAAGCAGGCAGCTGTTGATCC

[a] Recognition sites of restriction enzymes are given in bold; start methionine is underlined; replaced nucleotides are in italics. (s) sense; (as) antisense oligonucleotide.

2000 and Opti-MEM medium according to the manufacturer's instructions and selected in medium containing the appropriate antibiotic. Clonal cell lines were obtained from transfected cells by limiting dilution.

Immunoprecipitation of metabolically labeled chimeric α1-antitrypsin and Endo H treatment. For metabolic labeling of transfected clone 9 hepatocytes, [^{35}S]methionine (100 μCi/ml) and methionine-free DMEM was used. Clone 9 hepatocytes were chased at indicated time points and cells were lysed in PBS containing 1 % Triton X-100 and protease inhibitors. Chimeric α1-antitrypsin was immunoprecipitated from cell lysates and media and analyzed in 8 % SDS-polyacrylamide gels. Immunoprecipitated chimeric α1-antrypsin from cell lysates was treated with Endo H and PNGase F as previously described [27]. Radioactivity was visualized by fluorography after treatment with EN^{3}HANCER using X-Omatic AR film.

Protease protection assay and Western blot analysis. CHO-K1 cells expressing myc-tagged rat endomannosidase or rat endomannosidase$_{25}$-GFP were homogenized in 250 mM sucrose, 10 mM Tris-HCl pH 7.4 and centrifuged at 100 000 *g* for 1 h at 4°C. Membranes were incubated with 2.5 M urea on ice for 30 min and centrifuged as described above. Proteins of the pellet and supernatant were analyzed in 10 % SDS-polyacrylamide gels and subjected to Western blot analysis. For protease protection assay, urea-stripped membranes were treated with proteinase K (0.2 or 2 μg) in the absence or presence of 1 % Triton X-100. The reaction was stopped by the addition of trichloroacetic acid (TCA). The TCA-precipitated proteins were analyzed in 10 % and 12 % SDS-polyacrylamide gels, respectively, transferred onto nitrocellulose membranes using a semidry blotting apparatus [28]. The nitrocellulose membranes were blocked in PBS containing 1 % BSA for 1 h at ambient temperature and incubated with mouse anti-myc antibody and rabbit anti-GFP antibody, respectively, overnight at 4°C followed by the incubation with the corresponding horseradish-conjugated secondary antibody for enhanced chemiluminescence detection.
Western blot analysis of CHO-K1 cells expressing $rEndo_{25, 23, 21-9}$-GFP constructs was performed. CHO-

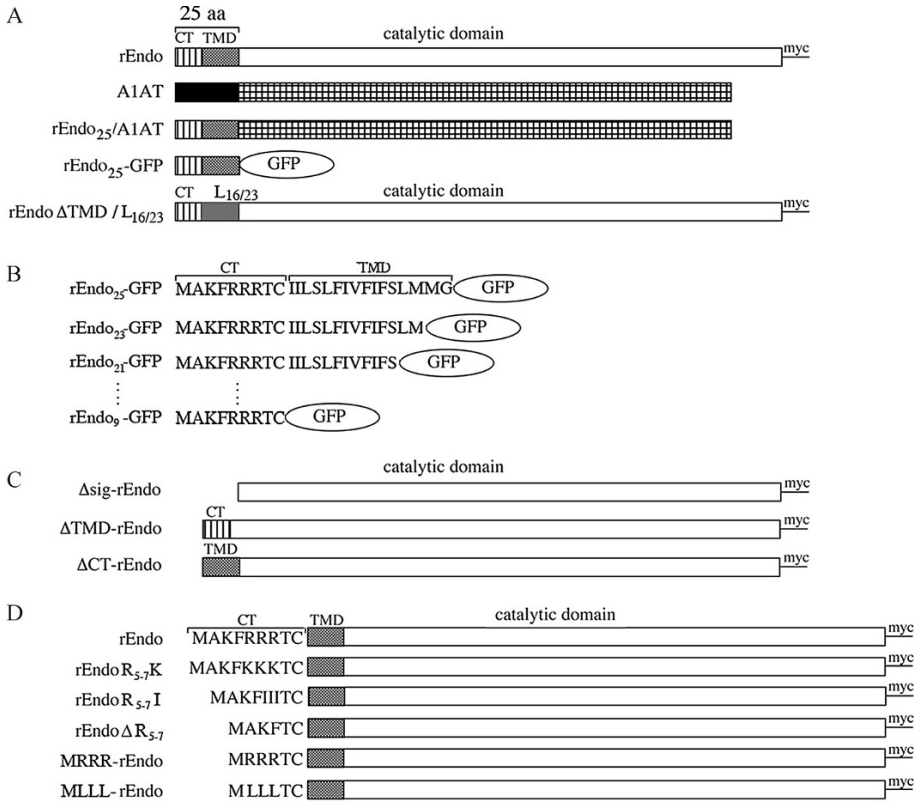

Figure 1. Schematic representation of various rat endomannosidase constructs used for transfections. (*A*) Myc-tagged rat endomannosidase with its putative signal sequence consisting of the cytoplasmic tail (CT) and the transmembrane domain (TMD) as well as the large luminal domain. The rat endomannosidase signal sequence was fused either to human α1-antitrypsin (A1AT) or to green fluorescent protein (GFP). Alternatively, the transmembrane domain of rEndo was completely replaced by poly-leucine, L_{16} and L_{23}, respectively. (*B*) The length of the transmembrane domain of $rEndo_{25}$-GFP was shortened from its C terminus to generate $rEndo_{23, 21, 19\ to\ 9}$-GFP constructs. The $rEndo_9$-GFP construct consists of the cytosolic tail of rEndo fused to GFP. (*C*) Myc-tagged rat endomannosidase lacking the whole signal sequence (Δsig-rEndo) or the transmembrane domain (ΔTMD-rEndo) or the cytoplasmic tail (ΔCT-rEndo). (*D*) Myc-tagged full-length rat endomannosidase (top row) and constructs with alterations in the cytoplasmic tail (lower rows).

K1 cells were homogenized and separated into a membrane and cytosolic fraction by differential centrifugation. Proteins were analyzed in 12 % SDS-polyacrylamide gels and subjected to Western blot analysis using a rabbit anti-GFP antibody (2 μg/ml) in combination with the corresponding horseradish-conjugated secondary antibody.

Confocal immunofluorescence microscopy. Transfected CHO-K1 cells grown as monolayer on glass cover slips were formaldehyde-fixed and saponin-permeabilized. Single (for α1-antitrypsin and for Man II, respectively) and double (myc and Man II or sec61β; α1-antitrypsin and Man II, respectively) immunolabeling incubations were performed as described [26]. Immunofluorescence was observed with Leica Confocal Laser Scanning Microscopes SP2 and SL2 using the 100× objective (1.4). In double-immunofluorescence overlays, effects of *z* axis pixel shifts were corrected.

Results

Rat endomannosidase is a type II membrane protein. Endomannosidase is located in *cis* and *medial* Golgi cisternae and to lesser amounts in pre-Golgi intermediates [23]. We have aimed to establish the molecular architecture of the enzyme and to identify putative Golgi retention targeting sequences. By stably expressing a myc-tagged rat endomannosidase (rEndo) in CHO-K1 cells, we observed that the enzyme behaved as a membrane-bound protein. Western blot analysis of CHO-K1 cell membranes, treated with urea to release membrane-associated proteins, showed two major anti-myc immunoreactive species in the membrane fraction, due to Golgi-associated post-translational modifications [26] and none in the supernatant (Fig. 2A). To obtain information about its membrane orientation, we determined sensitivity of endomannosidase against proteinase K. Treatment of CHO-K1 cell membranes with proteinase K did not alter rEndo, whereas Triton X-100

treatment of microsomes rendered it sensitive (Fig. 2B). In contrast, engineered rEndo lacking the cytoplasmic tail (ΔCT-rEndo) was sensitive to proteinase K (Fig. 2B), indicating the failure to acquire a type II membrane topology. Thus, rEndo possesses a type II membrane orientation containing a short N-terminal cytoplasmic tail, a transmembrane domain and a large catalytic domain situated in the lumen of the Golgi apparatus.

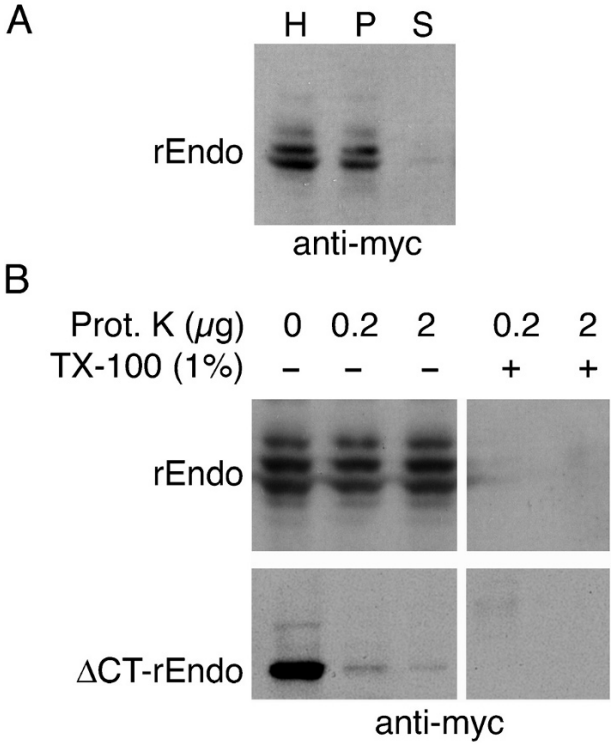

Figure 2. Rat endomannosidase is a type II membrane protein. (*A*) CHO cells stably expressing myc-tagged rat endomannosidase were homogenized (H) (see Materials and methods) and separated into pellet (P) and supernatant (S) by centrifugation at 100 000 *g* for 1 h followed by Western blotting using anti-myc antibody. (*B*) Urea-stripped membranes from CHO-K1 cells stably expressing myc-tagged rat endomannosidase (rEndo) were treated with proteinase K (0.2 and 2 μg) in the absence or presence of 1% Triton X-100 and analyzed by Western blotting with anti-myc antibody. The same procedure was applied to myc-tagged rat endomannosidase devoid of the cytoplasmic tail (ΔCT-rEndo).

The putative signal sequence of rat endomannosidase is sufficient for Golgi localization. Signal sequence prediction analysis of rEndo suggested a potential signal peptidase cleavage site between amino acid 25 and 26 [26]. However, no soluble form of endomannosidase was detectable (Fig. 2A), suggesting that the signal peptide is not cleaved. Thus, the transmembrane domain of rat endomannosidase ($rEndo_{25}$), included in the first 25 N-terminal amino acids, might act as membrane anchor and be responsible for Golgi retention of endomannosidase. To test this hypothesis, we constructed a chimeric α1-antitrypsin ($rEndo_{25}$/A1AT, Fig. 1A) containing the signal sequence of $rEndo_{25}$. When expressed in clone 9 hepatocytes (Fig. 3A–C) or in CHO-K1 cells (data not shown), the chimeric α1-antitrypsin showed a typical Golgi staining and overlapped with the immunofluorescence for Golgi mannosidase II. In contrast, human α1-antitrypsin expressed in clone 9 hepatocytes exhibited the reported ER staining pattern (Fig. 3D–F). Metabolic labeling showed that chimeric α1-antitrypsin was synthesized as a 52-kDa non-secreted glycoprotein carrying complex-type oligosaccharides as demonstrated by their Endo H resistance and PNGase F sensitivity (Fig. 4). Furthermore, expression of $rEndo_{25}$ fused to theGFP ($rEndo_{25}$-GFP, Fig. 1A) in CHO-K1 cells (Fig. 3G–I), HepG2 and HeLa cells (data not shown) resulted in Golgi localization. Hence, the 25 N-terminal amino acids of rEndo, which include the cytoplasmic tail and the transmembrane domain, appear to contain the structural information for Golgi retention of the enzyme.

The cytoplasmic tail and transmembrane domain of rat endomannosidase are required for its Golgi localization. The Golgi localization of various glycosyltransferases is dependent on the length of their hydrophobic region [15, 29]. To test whether this observation applies to endomannosidase, various rEndo-GFP constructs were made, in which the length of the transmembrane domain was shortened from its C terminus ($rEndo_{23,\ 21,\ 19–11}$-GFP, Fig. 1B) or which were devoid of it ($rEndo_{9}$-GFP, Fig. 1B). When stably expressed in CHO-K1 cells, all studied rEndo-GFP constructs with a signal sequence <25 amino acids, *i.e.*, a transmembrane domain with <16 amino acids, showed a diffuse cytoplasmic staining (Fig. 5A). Membrane and cytosolic fractions of CHO cells expressing the various rEndo-GFP constructs were analyzed by Western blotting. Only the $rEndo_{25}$-GFP was detectable in the membrane fraction, whereas $rEndo_{23}$-GFP, $rEndo_{21}$-GFP and GFP alone were mainly found in the cytosolic fraction (Fig. 5B) similar to all other analyzed rEndo-GFP constructs (data not shown). Furthermore, rEndo was Golgi-retained if its transmembrane domain was replaced by 16 leucine residues (Fig. 3K–M). If the length of the poly-leucine stretch was increased to 23 leucine residues, a vesicular staining pattern and occasionally a faint cell surface staining was observed beside the Golgi staining (Fig. 3N–P). Collectively, these data indicate that the length of the transmembrane domain of rEndo is important for Golgi localization.

Next, the importance of each domain of rEndo for Golgi localization was evaluated. For this we gener-

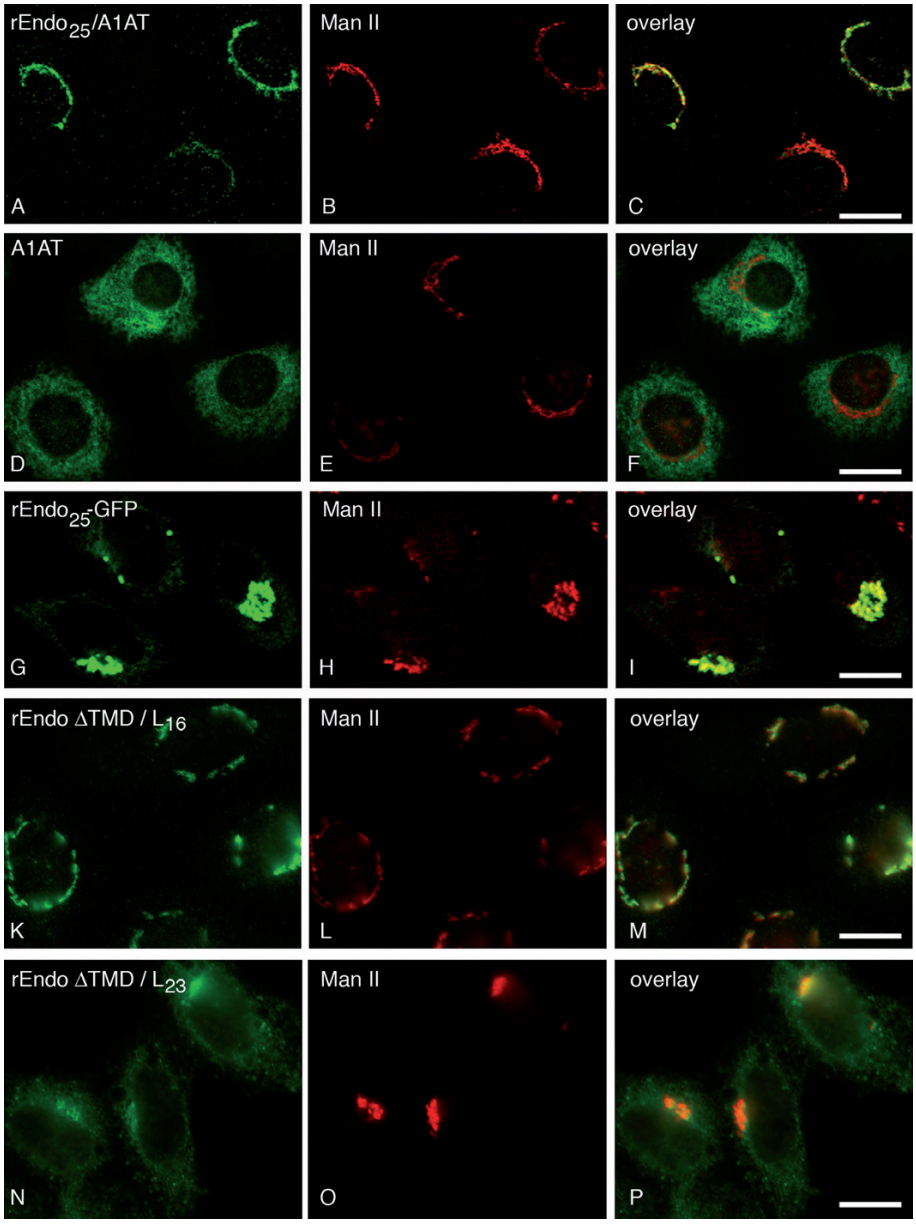

Figure 3. Endomannosidase signal sequence is sufficient for Golgi localization of α1-antitrypsin and GFP. Confocal double immunofluorescence for α1-antitrypsin and endogenous Golgi-mannosidase II in clone 9 hepatocytes. In a single confocal section, $rEndo_{25}$/α1-antitrypsin (*A*) and Golgi mannosidase II (*B*) showed co-distribution (*C*), whereas wild-type human α1-antitrypsin (*D*) only partially co-localized with Golgi mannosidase II (*E*, *F*). $rEndo_{25}$-GFP (*G*) stably expressed in CHO-K1 cells and Golgi-mannosidase II (*H*) showed co-distribution (*I*). rEndo with a transmembrane domain of 16 leucine residues, rEndo $\Delta TMD/L_{16}$, (*K*) and Golgi-mannosidase II (*L*) showed co-distribution (*M*) in CHO-K1 cells, whereas rEndo with a transmembrane domain of 23 leucine residues, rEndo $\Delta TMD/L_{23}$ (*N*) showed a broader subcellular distribution (*P*). Bars, 10 μm.

ated myc-tagged rEndo constructs (Fig. 1C) where the entire signal sequence was deleted (Δsig-rEndo), or either the 16 amino acids of the transmembrane domain (ΔTMD-rEndo) or the cytoplasmic tail (ΔCT-rEndo) were missing. In CHO-K1 cells expressing Δsig-rEndo or ΔTMD-rEndo, endomannosidase was undetectable by confocal microscopy. In agreement with this finding, no rEndo DNA fragment could be amplified by RT-PCR using RNA from the transfected CHO-K1 cells and rEndo-specific primers. On the other hand ΔCT-rEndo exhibited an ER localization (Fig. 6A–C) and failed to maintain a type II membrane topology (Fig. 2B).

Positively charged amino acids in the cytoplasmic tail of endomannosidase are not essential for Golgi localization. Remarkably, vertebrate endomannosidases and CHO-K1 cell endomannosidase [26] possess a cluster of conserved positively charged amino acid in their cytoplasmic tail (Table 2). To evaluate their requirement for Golgi localization, we replaced or deleted these residues (Fig. 1D). The myc-tagged rEndo $R_{5-7}K$ exhibited a typical Golgi localization (data not shown), similarly to a back-engineered Cys177/Cys188Trp CHO cell endomannosidase possessing two arginine and one lysine residue at this position [26]. Surprisingly, rEndo $R_{5-7}I$ (Fig. 6D–F) or rEndo $R_{5-7}L$ (data not shown) also exhibited a Golgi localization, whereas rEndo ΔR_{5-7} was ER localized

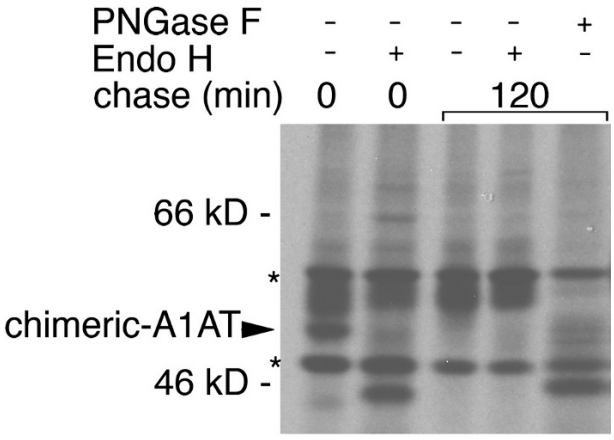

Figure 4. Intracellular retained chimeric α1-antitrypsin is carrying complex type oligosaccharides. Clone 9 rat hepatocytes expressing chimeric α1-antitrypsin were pulsed for 20 min and chased for the time period indicated. Cell lysates was immunoprecipitated with anti-human α1-antitrypsin antibody and analyzed by 8% SDS-PAGE/fluorography. The intracellular chimeric α1-antitrypsin was Endo H-resistant and PNGase F sensitive. The asterisks indicate co-immuoprecipitated proteins.

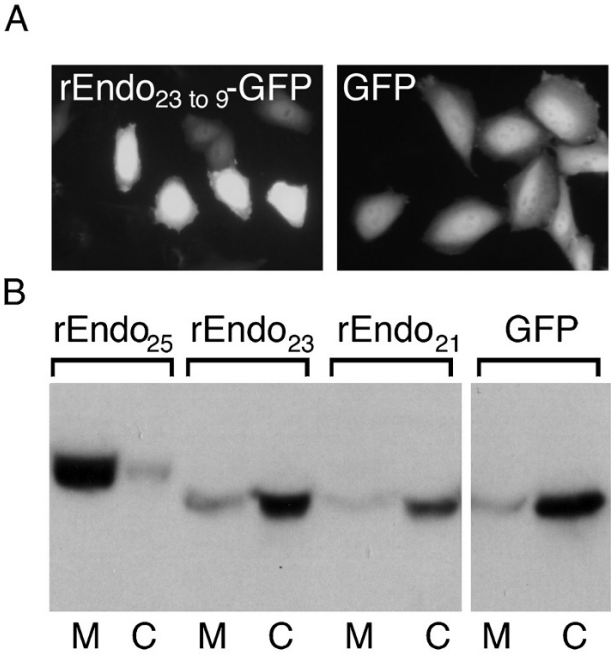

Figure 5. Rat endomannosidase-GFP constructs with a shortened signal sequence showed cytoplasmic localization. (*A*) CHO-K1 cells expressing GFP fused to a truncated signal sequence of endomannosidase ($rEndo_{23}$ to $rEndo_9$) exhibited a cytoplasmic staining similar to GFP alone. (*B*) CHO-K1 cells, stably expressing GFP fused to rat endomannosidase signal sequence of various length, were separated into a membrane (M) and cytosolic fraction (*C*) by differential centrifugation (see Materials and methods) and analyzed by Western blotting using anti-GFP antibody.

(data not shown). Furthermore, rEndo with a truncated cytoplasmic tail possessing three arginine residues (MRRR-rEndo) showed a typical Golgi localization and no ER staining was observed (Fig. 6G–I). Moreover, rEndo with the same length of cytoplasmic tail containing three leucine residues (MLLL-rEndo) instead of arginines also exhibited a Golgi localization (Fig. 6K–M). Next, specific positively amino acid residues in the cytoplasmic tail of rEndo were either replaced by neutral ones or completely deleted (Table 3) to determine the contribution of each of this charged residue for Golgi localization. The subcellular distribution of engineered $rEndo_{25}$-GFP constructs, determined by immunofluorescence microscopy, was consistent with those of full-length rat endomannosidase containing the corresponding mutations (Table 3). Replacement of K_3, or one of the three consecutive arginine residues, *e.g.*, R_5, R_6 or R_7, by leucine did not alter the inherent Golgi localization (Table 3). Similarly, $rEndo_{25}R_{5-7}$I-GFP (Fig. 7A–C) or $rEndo_{25}R_{5-7}$L-GFP, (Fig. 7D–F) as well as $rEndo_{25}R_{5-7}$ H-GFP (data not shown) were Golgi-retained and maintained a type II topology (Fig. 8). Thus, the presence of positively charged amino acids in the cytoplasmic tail of endomannosidase is not essential for its Golgi localization as long as the engineered protein achieves a type II topology.

Discussion

In the present study, we have experimentally shown that Golgi endomannosidase possesses as a type II membrane protein architecture. Furthermore, we have identified specific amino acids important for correct topology and putative targeting sequences responsible for Golgi retention of endomannosidase. Protease protection experiments (present study) and susceptibility against detergents [27, 30] indicated that endomannosidase is an integral type II membrane protein containing a short cytoplasmic tail, a single transmembrane domain and a large, luminal-located catalytic domain. Thus, its architecture and amino acid composition of the transmembrane domain resemble those of other Golgi-localized enzymes [17, 31, 32].
Our biochemical and microscopical data demonstrated that the 25 N-terminal amino acids of rat endomannosidase, comprising the cytoplasmic tail and the transmembrane domain are sufficient to retain two reporter proteins, the secretory α1-antitrypsin or GFP in the Golgi apparatus. Golgi retention was prevented by reducing the length of the transmembrane domain of endomannosidase, as reported for sialyltransferase [15, 29] and for syntaxins [33]. On the other hand, replacement of the transmembrane domain by 16 leucine residues resulted in Golgi localization of rat endomannosidase, while increasing the length to 23 leucine residues reduced its Golgi retention. Thus, the

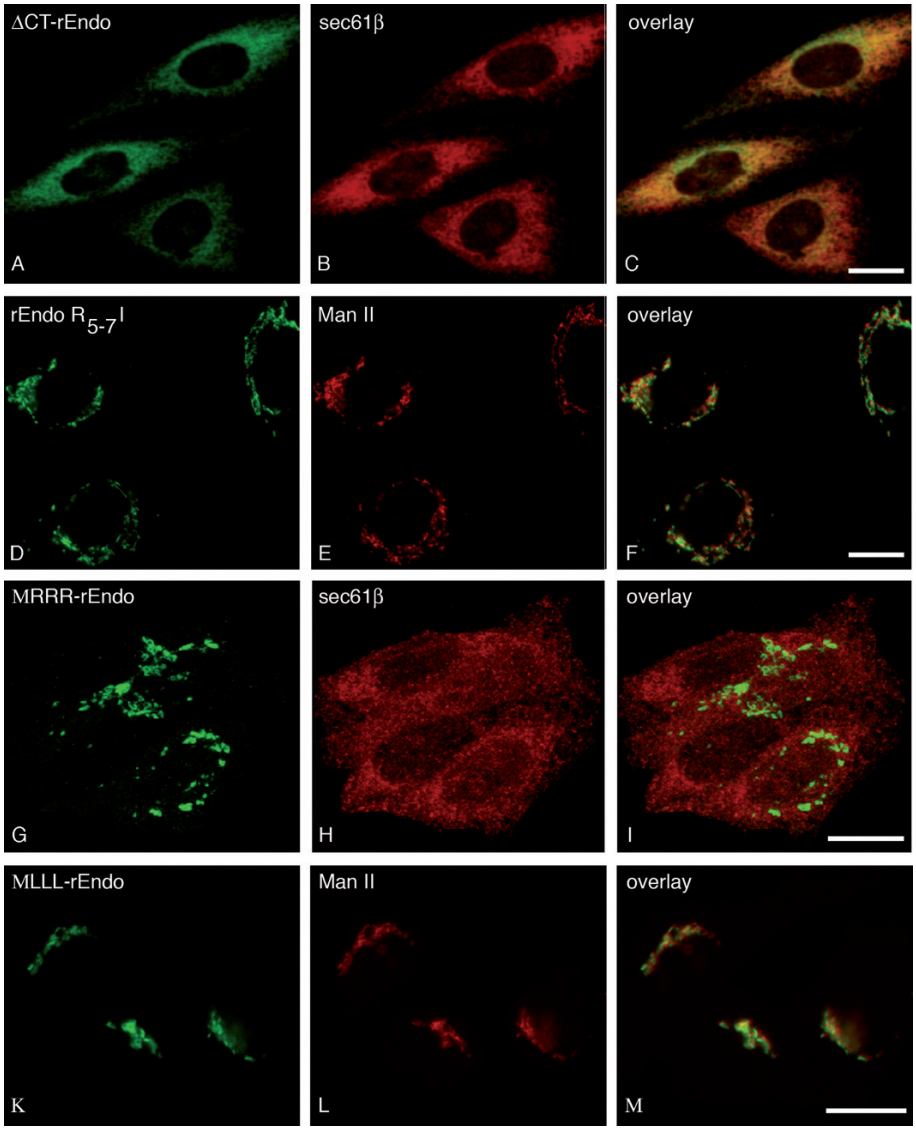

Figure 6. Rat endomannosidase possessing positively charged amino acids within the cytoplasmic tail exhibited Golgi localization. Confocal double immunofluorescence for myc-tagged rat endomannosidases expressed in CHO-K1 cells and Golgi mannosidase II or ER marker Sec61β, respectively. Rat endomannosidase lacking the cytoplasmic tail ΔCT-rEndo (*A*), overlapped with Sec61β (*B*, *C*). Engineered rat Endo $R_{5-7}I$ (*D*) and Golgi mannosidase II (*E*) showed co-distribution (*F*). Rat endomannosidase with a truncated cytoplasmic tail possessing three arginines, MRRR-rEndo (*G*) and Sec61β (*H*) did not overlap (*I*). Rat endomannosidase with truncated cytoplasmic tail possessing three leucine residues instead of arginines, MLLL-rEndo (*K*), and Golgi mannosidase II (*L*) showed co-distribution (M). Bars, 10 μm.

Table 2. Highly conserved positively charged amino acids in the cytoplasmic tail of endomannosidase of various species.

Cytoplasmic tail[a]	TMD sequence	Accession no.[b]	Organism
MA**K**F**RRR**TC	IILSLFIVFIFSLMMG	NP_542963	*Rattus norvegicus*
MA**K**F**RRR**TC	IILALFIVFIFSLMMG	NP_078917	*Homo sapiens*
MA**K**F**RRR**TC	IILALFILFIFSLMMG	XP_001096615	*Macaca mulatta*
MA**K**F**RRR**TC	ILLSLFILFIFSLMMG	NP_766453	*Mus musculus*
MA**K**F**RRR**TC	IILSLIILFIFSLMMG	XP_539046	*Canis familiaris*
MA**K**F**RR**GTC	IILALFILFIFSLMMG	Q5RD93	*Pongo pygmaeus*
MA**R**F**RRR**TC	IILSLFILLICSLMMG	XP_001377143	*Monodelphis domestica*
MA**R**F**RRR**TC	IILLLFILFICSIMMG	XP_419831	*Gallus gallus*
MI**R**F**RRR**TC	ITLSIFIFLVCLIM	NP_001086682	*Xenopus leavis*
MA**K**F**RRK**TC	IILSLFILFIFSLMMG	DQ825405	*Cricetulus griseus*

[a] The positively charged amino acids in the cytoplasmic tail are in bold.
[b] GenBank accession number.

Table 3. Subcellular localization rat endomannosidase$_{25}$-GFP constructs with mutations in the cytoplasmic tail[a].

Construct[b]	CT	TMD	Localization
rEndo$_{25}$	MAKFRRRTC	IILSLFIVFIFSLMMG	Golgi
MRRR-rEndo$_{25}$	MRRRTC	IILSLFIVFIFSLMMG	Golgi
ΔCT-rEndo$_{25}$	M	IILSLFIVFIFSLMMG	ER
rEndo$_{25}$ΔR$_{5-7}$	MAKFTC	IILSLFIVFIFSLMMG	ER
rEndo$_{25}$K$_3$L	MALFRRRTC	IILSLFIVFIFSLMMG	Golgi
rEndo$_{25}$R$_5$L	MAKFLRRTC	IILSLFIVFIFSLMMG	Golgi
rEndo$_{25}$R$_6$L	MAKFRLRTC	IILSLFIVFIFSLMMG	Golgi
rEndo$_{25}$R$_7$L	MAKFRRLTC	IILSLFIVFIFSLMMG	Golgi
rEndo$_{25}$R$_{5-7}$I	MAKIIITC	IILSLFIVFIFSLMMG	Golgi
rEndo$_{25}$R$_{5-7}$L	MAKFLLLTC	IILSLFIVFIFSLMMG	Golgi
rEndo$_{25}$R$_{5-7}$H	MAKFHHHTC	IILSLFIVFIFSLMMG	Golgi

[a] CT, cytoplasmic tail; TMD, transmembrane domain.
[b] The generation of the various rat endomannosidase-GFP constructs is described under Materials and methods.

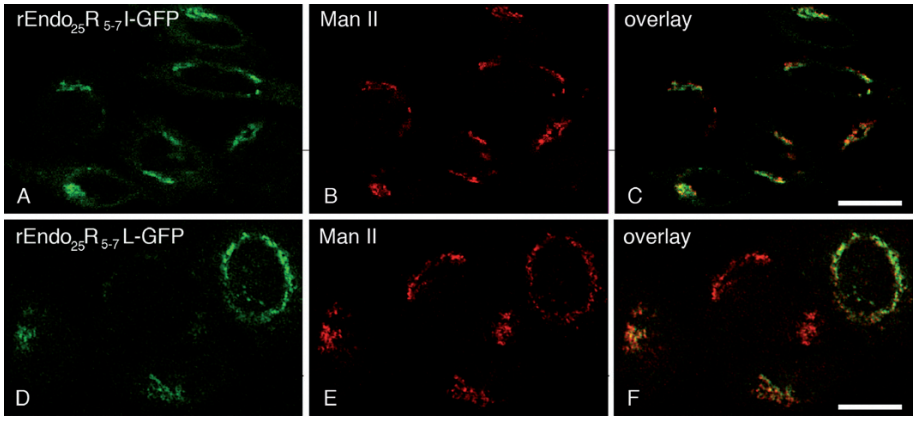

Figure 7. Positively charged amino acids within the cytoplasmic tail of rat endomannosidase$_{25}$-GFP are not essential for Golgi localization. Double confocal immunofluorescence for rEndo$_{25}$R$_{5-7}$I-GFP (*A*) or rEndo$_{25}$R$_{5-7}$L-GFP (*B*) and Golgi-mannosidase II (*B*, *E*) showed overlapping distributions (*C*, *F*). Bars 10 μm.

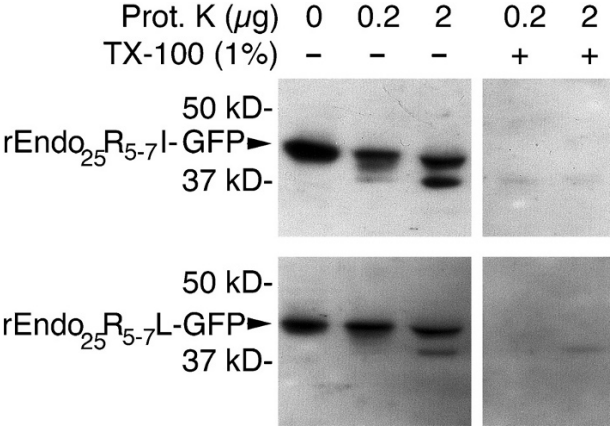

Figure 8. Rat endomannosidase$_{25}$-GFP devoid of positively charged amino acid in the cytoplasmic tail maintains type II topology. Urea-stripped membranes from CHO-K1 cells expressing rEndo$_{25}$R$_{5-7}$I-GFP or rEndo$_{25}$R$_{5-7}$L-GFP were treated with proteinase K (0.2 and 2 μg) in the absence or presence of 1 % Triton X-100 and analyzed by Western blotting with anti-GFP antibody.

length of the transmembrane domain has a major effect in Golgi retention of the endomannosidase. Similarly, the importance of the transmembrane domain for the localization of other Golgi resident enzymes has been demonstrated [29, 34–37]. Of course, the possibility remains that the cytoplasmic tail of endomannosidase is also involved in Golgi retention, as reported for the Golgi retention of fucosyltransferase [19, 32]. Collectively, we conclude that endomannosidase Golgi retention occurs *via* the bilayer thickness mechanism. This notion was further supported by the fact that we did not obtain direct evidence for specific interaction or kin recognition [21] between endomannosidase and other proteins of the *cis* or *medial* Golgi. Moreover, it was shown that the kin recognition between enzymes of the *medial* Golgi involves luminal sequences of the enzyme rather than transmembrane domains [15, 18].

Endomannosidases from various species share as distinguishing feature a cluster of arginine residues in their cytoplasmic tail (Table 2). Multiple arginine residues located at specific positions of the N terminus

were responsible for ER targeting of some type II membrane proteins [8, 38], similar to the double lysine motif found at the C terminus of type I membrane proteins [4–7]. Conversely, the localization of endomannosidase, a type II membrane protein, excludes the possibility that these arginine residues constitute an ER-targeting motif. Thus, depending on the position of the arginine residues within the cytoplasmic tail, they act as ER retention or ER export signal. So far, a di-acidic motif located at variable distance from C terminus of some transmembrane secretory proteins has been proposed as an ER export signal [39–42], whereas ER export of proteins cycling between ER and Golgi depends on hydrophobic residues in their cytoplasmic tail [43, 44]. More recently, a novel type of ER export signal has been described consisting of dibasic amino acids, located proximal to the transmembrane domain in the cytoplasmic tail of Golgi-resident glycosyltransferase that is required for type II membrane protein to exit the ER by COPII vesicles [45]. Indeed, full-length rat endomannosidase possesses three consecutive arginine residues proximal to the transmembrane domain. Based on our data, the arginine at position 5 and 6, 6 and 7 as well as 5 and 7 can be part of the dibasic motif. Furthermore, the highly conserved arginine residues in endomannosidase can be fully replaced by other positively charged amino acids such as lysine residues as well as a combination between arginine/lysine as found in engineered CHO cell endomannosidase [26] without affecting its intracellular localization. Surprisingly, substitution of the positively charged amino acid residues in the cytoplasmic tail residues by neutral amino acids did not abolish Golgi localization. Thus, in addition to the positive charge, the structural information of the amino acid side chain contributes to topology resulting in ER to Golgi transport competence. Therefore, properly oriented mutagenized proteins reach the Golgi, whereas those with inverted topology, like the cytoplasmic tail-deleted endomannosidase, remains in the ER.

Removal of glucose residues from the *N*-linked oligosaccharides precursor occurs either by the classical trimming pathway by glucosidases or by the alternate endomannosidase pathway. Glucosidase I is retained in the ER by sequence motifs located in the luminal domain [38] and glucosidase II by the so-called β-subunit carrying the C-terminal KDEL retrieval sequence [46–48], whereas endomannosidase is retained in the Golgi by its 25 N-terminal amino acids. Thus, distinct mechanisms ensure that the glucose-trimming enzymes are located in different compartments [23, 49] and fulfill specific functions. ER-located glucosidase II is not only involved in the biosynthesis of *N*-linked oligosaccharides but is also a key enzyme in the quality control of glycoprotein folding [50, 51], whereas endomannosidase provides a back-up mechanism for protein *N*-glycosylation in the Golgi apparatus [27]. Furthermore, the Golgi localization of endomannosidase allows a quality control of glycoprotein folding and assembly by the calnexin/calreticulin cycle [51].

In summary, we elucidated the molecular structure of endomannosidase and demonstrated that both the cytoplasmic tail and the transmembrane domain of endomannosidase are necessary for efficient Golgi retention of the enzyme. The former one is required for correct topology, whereas the latter is necessary for the Golgi retention.

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